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BRIEF COMMUNICATION

Application of fluorimetric analysis of plant esterases to study of programmed cell death and effects of cadmium(II) ions

J. VÍTEČEK[1], J. PETRLOVÁ[2], J. PETŘEK[1], V. ADAM[2,3], L. HAVEL[1], K.J. KRAMER[4] and R. KIZEK[2]*

Department of Plant Biology[1], and Department of Chemistry and Biochemistry[2], Mendel University of Agriculture and Forestry, Zemědělská 1, CZ-61300 Brno, Czech Republic.
Department of Analytical Chemistry, Faculty of Science, Masaryk University, Kotlářská 2, CZ-61137 Brno, Czech Republic[3]
Grain Marketing and Production Research Center, Agricultural Research Service, US Department of Agriculture, Manhattan, KS 66502, USA[4]

Abstract

Esterases (EC 3.1.1.x) represent a diverse group of hydrolases catalyzing the cleavage and formation of carboxyl ester bonds. Their connection with development has made them a suitable marker of development in plants. In the present work, we focused on the fluorimetric determination of the plant esterases in plant cell cultures (tobacco BY-2 cells and early somatic embryos of Norway spruce, clone 2/32) with respect to application the method for the study of programmed cell death and the influence of cadmium(II) ions on the plant cells. The programmed cell death has been triggered by sodium nitroprusside and glucose oxidase. The determination of the esterase activity by the proposed technique in a cell extract determined very small difference in enzyme activity, which was a reliable marker of metabolic changes. In addition, the esterase activity of spruce somatic embryos decreased with the increase in medium Cd concentration.

Additional key words: attomole detection, confocal microscopy, early somatic embryos of Norway spruce, fluorescein diacetate, fluorescence microscopy, protoplast, tobacco BY-2.

Esterases (EC 3.1.1.x) represent a diverse group of hydrolases catalyzing the cleavage and formation of carboxyl ester bonds. They are involved in formation of cell wall (Willats *et al.* 2001), degradation of some xenobiotics (Sandermann 1992, Cummins *et al.* 2001) and signalling (Stuhlfelder *et al.* 2002). Esterases have been used as a marker of viability in combination with other dyes for long time (Jones and Senft 1985). Furthermore their connection with development has made them a suitable marker of development in plants. Due to complex isoenzyme pattern of esterases and specifity for individual species or varieties they have also been suggested for the purpose of their identification (Krulíčková *et al.* 2002, Tsanev *et al.* 2004, Stoilova *et al.* 2006).

Recently we have shown that plant esterases could also serve as growth marker of different plant cell cultures (Víteček *et al.* 2004, 2005). That approach is capable of replacing methods based on the counting and/or weighing of cells or tissues because of low demands on the sample size. In the present work, we focused on the optimization of the fluorimetric determination of the plant esterases in plant cell cultures: tobacco BY-2 cells and early somatic emryos (ESEs) of Norway spruce clone 2/32. Further we applied the method for the study of programmed cell death and the influence of cadmium on plant cells.

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Abbreviations: BA - N^6-benzyladenine; Cd-EDTA - cadmium-ethylenediaminetetraacetic acid chelate, 2,4-D - 2,4-dichlorophenoxyacetic acid; ESEs - early somatic embryos, FDA - fluorescein diacetate, GGO - glucose oxidase, IU - international unit, NO - nitric oxide, PCD - programmed cell death, PE - plant esterases, PI - propidium iodide, SNP - sodium nitroprusside.
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* Author for correspondence: fax: (+420) 545 212 044, e-mail: kizek@sci.muni.cz

Fluorescein diacetate (FDA), propidium iodide (PI), pig liver esterase (suspension in 3.2 M ammonium sulphate) and all other used reagents were purchased from *Sigma-Aldrich* (St. Louis, USA), unless noted otherwise. Celulysin was purchased from *Calbiochem* (San Diego, USA). All solutions were prepared using deionised water. If not otherwise indicated, culture media were prepared using plant cell culture chemicals purchased from *Duchefa Biochemie* (Haarlem, The Netherlands).

A suspension culture of *Nicotiana tabacum* line BY-2 was grown in liquid Murashige and Skoog (1962; MS) medium supplemented with sucrose (30 g dm^{-3}), KH_2PO_4 (0.2 g dm^{-3}), thiamine (1 mg dm^{-3}) and 2,4-dichlorophenoxyacetic acid (2,4-D; 0.2 mg dm^{-3}), according to Nagata *et al.* (1992). The suspension cultures (20 cm^3) were grown in 50 cm^3 Erlenmeyer flasks at 27 °C with shaking at 135 min^{-1} (type *LT-W*, *Adolf Kühner AG*, Birsfelden, Switzerland). Subcultivation was performed after 3 or 4 d by transferring 2 or 1 cm^3, respectively, of suspension culture into a fresh medium (total volume 20 cm^3). Counting of BY-2 cells was carried out using a *Fuchs-Rosenthal* haemocytometer (*Glaswarenfabrik Karl Hecht KG*, Sondheim, Germany).

A culture of early somatic embryos (ESEs) of *Picea abies* (L.) Karst. clone 2/32 was used. The clone was originally established at the Mendel University of Agriculture and Forestry in Brno, Czech Republic, according to a procedure described by Durzan *et al.* (1994) and Jokinen and Durzan (1994). The ESEs were maintained on a semisolid (*Gelrite*, *Merck*, Prague, Czech Republic), half-strength LP medium (Von Arnold 1987) modified by Havel and Durzan (1996ab). The concentration of 2,4-D and N^6-benzyladenine (BA) was 4.4 and 9 μM, respectively. The pH was adjusted to 5.7 - 5.8 before autoclaving (121 °C, 100 kPa, 20 min). The organic part of the medium, except sucrose, was sterilized by filtration through a 0.2 μm polyethylensulfone membrane (*Puradisc 25 AS*, *Whatman*, Clifton, USA). Ten ESEs clusters were cultivated in one plastic Petri dish (100 mm in diameter) containing 30 cm^3 of the medium. Sub-cultivation of stock cultures was carried out at two-week intervals. The stock and experimental cultures were maintained in a cultivation box in the dark at temperature of 23 ± 2 °C.

A modified double-staining method with fluorescein diacetate (FDA) and propidium iodide (PI) was used for the determination of the viability of BY-2 cells and ESEs (Jones and Senft 1985). FDA penetrates easily into viable cells, where it is hydrolysed by esterases to fluorescein (green fluorescence). The red fluorescence of PI in cells shows that these cells are dead, because this compound cannot pass through the functional cytoplasmic membrane. In our experiments, about 1 mg of plant material was harvested and diluted with water to a final volume of 0.05 cm^3. The stock solutions of PI and FDA were added to a final concentration of 20 and 1 μg cm^{-3} respectively (Mlejnek and Procházka 2002). After 5 min of incubation at room temperature, the percentage of dead (red-stained cells) and viable cells (green-stained cells) was evaluated using an *Olympus* (Prague, Czech Republic) *AX 70* fluorescence microscope connected to a digital camera (*Olympus 4040 Zoom*). Areas of dead (red staining) and viable cells (green staining) were monitored by means of image analysis (IA; *Image-Pro program*, *ver. 1.3*, *Sony*, San Diego, USA) (Petřek *et al.* 2005).

Cultivation medium from tobacco BY-2 cells was removed by centrifugation (360 *g*; 5 min; 20 °C; *MR 22*, *Jouan*, Saint-Herblain, France) (Víteček *et al.* 2004). The cells were washed twice in 50 mM potassium phosphate buffer (pH 8.7). The washed BY-2 cells and harvested ESEs (100 - 200 mg) were mixed with extraction buffer (250 mM potassium phosphate, pH 8.7) to a final volume of 1 cm^3 and homogenised using a Potter-Elvehjem homogenizer (*Kavalier*, Sázava, Czech Republic) placed in an ice bath for 10 min. The redox state of the obtained solution was maintained by addition of dithiothreitol (DTT) at a concentration of 1 mM. The homogenised samples were sonicated for 1 min in an ice bath using a *Transsonic T310* sonicator (*Elma*, Singen, Germany). The homogenate was centrifuged at 10 000 *g* for 15 min at 4 °C and an aliquot (0.005 - 0.020 cm^3) of the supernatant was mixed with 1 M potassium phosphate buffer (pH 8.7). The reaction was started by the addition of FDA to a final concentration of 5 μM and the final volume of the reaction mixture was 1 cm^3. An equal volume of extraction buffer was used as a blank. After incubation for 15 min at 45 °C, an aliquot (0.01 cm^3) of the reaction mixture was added to 1.99 cm^3 of 250 mM potassium phosphate buffer (pH 8.7, 25 °C). The fluorescence ($\lambda_{excitation}$ 490 nm and $\lambda_{emission}$ 514 nm) was read immediately using a spectrofluorimetric detector (*RF-551*, *Shimadzu*, Duisburg, Germany). A stock solution of FDA was prepared in acetone dried by anhydrous calcium chloride. The amount of acetone did not exceed 1 % (v/v) in the reaction mixture. Esterase activity is expressed in international units (IU, one IU liberates 1 μmol of fluorescein per minute under above specified conditions)

A stationary suspension culture of tobacco BY-2 cells was diluted 4:100 (v/v) with fresh cultivation medium. At the 3rd day of cultivation, sodium nitroprusside (SNP, donor of nitric oxide) glucose with glucose oxidase (GGO, donor of H_2O_2) were added to final concentration of 0.5 mM and 0.5 mM with 0.5 IU cm^{-3}, respectively (de Pinto *et al.* 2002). Besides that BY-2 cell suspension prepared as well as ESEs were treated by cadmium-ethylenediaminetetraacetic acid chelate (Cd-EDTA). A stock solution of Cd-EDTA was prepared by mixing $Cd(NO_3)_2$ with ethylene diamine tetra-acetic acid in a 1:1 molar ratio and stirred at 50 °C for 1 h. The filter-sterilized Cd-EDTA was added into the cell suspension of BY-2 cells prepared as above or autoclaved culture medium for cultivation of 2/32 ESEs. Viability and esterase activity was assayed after 24 h (BY-2 cells) or 120 h (2/32 ESEs), respectively.

The data acquired were processed in *MS Excel* and *Statgraphics* was used for statistical analyses. Results are

expressed as the mean values ± SE unless noted otherwise. A value of $P < 0.05$ was considered significant.

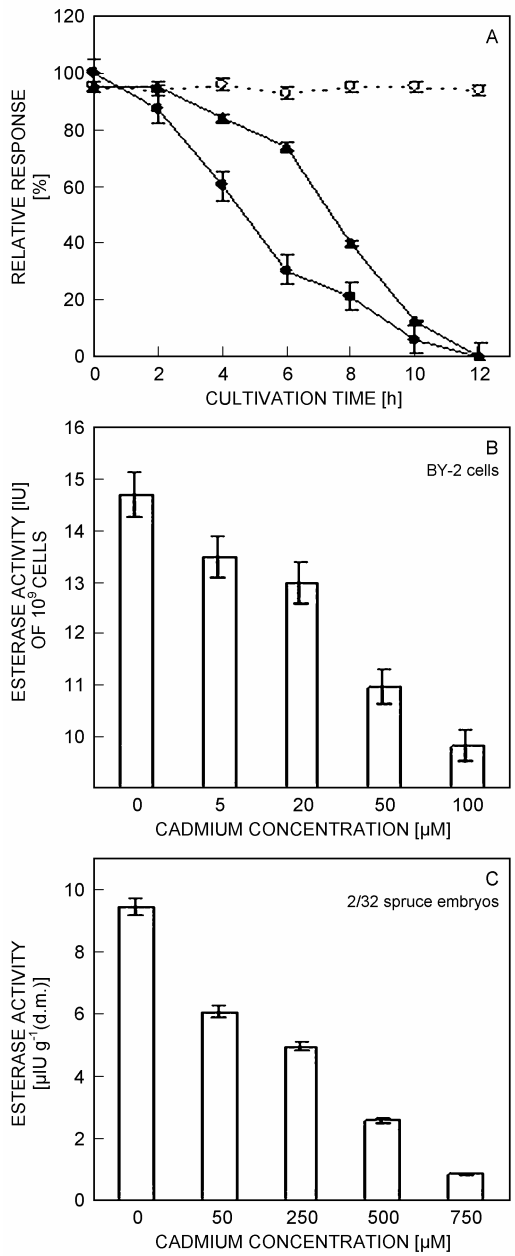

Fig. 1. *A* - Effect of NO and H_2O_2. Time course of viability (*triagles*) and intracellular esterase activity (*closed circles*) of BY-2 tobacco cells treated 0.5 mM sodium nitroprusside and 0.5 mM glucose with 0.5 IU cm^3 of glucose oxidase and viability of non-treated cells (control, *open circles*). *B*, *C* - Effect of cadmium (Cd-EDTA) on esterase activity of BY-2 tobacco cells (*B*) and 2/32 early somatic embryos (*C*), after 24 h (*B*) or 120 h (*C*) of treatment. Data are means ± SE ($n = 3$).

Nitric oxide serves in plants as a signal molecule that markedly influences the expression of a number of genes and also participates in programmed cell death (PCD; Neill *et al.* 2002). Recently, it was published that a mutual effect of hydrogen peroxide (H_2O_2) and nitric oxide (NO) is necessary for triggering of PCD during the hypersensitive response of plants (Delledonne *et al.* 2002). We were interested in whether we would be able to study cell death by means of the measurements of esterase activity. Neither SNP nor GGO induced any decrease of the viability of tobacco cells during 12 h experiments. On the other hand if we added SNP and GGO together, we observed a dramatic effect on the viability of tobacco cells (Fig. 1*A*) as reported previously (Delledonne *et al.* 2002, De Pinto *et al.* 2002). NO and H_2O_2 lead to PCD, and after 12 h, all cells were dead. The result of double staining with FDA/PI was different from the *in vitro* assay for PE (Fig. 1*A*). Assessment of the viability at the single cell level represented only a qualitative characteristic, whether the cell was alive or dead. On the other hand, the determination of the esterase activity by the proposed technique in a cell extract determined very small difference in enzyme activity, which was a reliable marker of metabolic changes.

We also tested the method for esterase determination to evaluate the influence of heavy metals on cell cultures. The effects of heavy metals on cell biology are a very active area of research (Kizek *et al.* 2002, Vacek *et al.* 2003, Potěšil *et al.* 2005, Wojcik and Tukiendorf 2005, Österås and Greger 2006). Their influence was studied in plants from two different points of view, detoxification [ability to survive in the presence of toxic heavy metal concentration (Ahearn *et al.* 2004, Gupta *et al.* 2004, Klejdus *et al.* 2004)] and biotechnology [hyperaccumulative plant for removal of heavy metals from the environment (Chen *et al.* 2004, Qadir *et al.* 2004, Suresh and Ravishankar 2004, Zehnálek *et al.* 2004)]. In our experiments, we added cadmium in a chelated form to tobacco cells and ESEs cultivation medium (Vacek *et al.* 2003). Tobacco cell cultures were incubated with 0 to 100 μM Cd-EDTA for 24 h. Viability decreased by more than 30 % under treatment with 100 μM Cd-EDTA (Fig. 1*B*). The results were in good agreement with the results obtained by the commonly used FDA/PI double staining method. In the case of embryonic cultures growing on semisolid medium, higher heavy metal concentrations were needed to see the toxic effect. The effect of different Cd-EDTA concentrations (0 to 750 μM) on ESEs after 120 h long treatment is shown in Fig. 1*C*. Only 11 % of the cells from the initial number survived at the highest Cd-EDTA concentration at the end of the experiment. In the embryonic cell culture, the assessment of viability is very difficult using the standard technique of FDA/PI double staining, because the cellular structure of the embryos is very complex. That is why the proposed method for the detection of esterase activity may serve as a reliable tool for viability detection in this model system.

The analytical method developed for the quantification of esterases, based on the fluorimetric detection of the products of enzymatic fluorescein diacetate

hydrolysis, represents a new and very sensitive tool for the study of growth and metabolic changes of cell cultures in a commonly used plastic cuvette. In addition, the method enables to study the growth characteristic of cultures that form solid clusters of cells. The method can be used for environmental analysis of the influence of stress factors on living systems.

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Van Alfen, N.K., Bruening, G., Dawson, W.O. (ed.): **Annual Review of Phytopathology. Vol. 44. -** Annual Reviews, Palo Alto 2006. 538 pp. Individual price USD 80.00. ISBN 0-8243-1344-5.

This is a new volume of the well known series that traditionally gives an overview of various fields of phytopathology. The introductory chapter written by S. Ouchi is devoted to retrospective of plant pathology. Burdon *et al*. in chapter "The Current and Future Dynamics of Disease in Plant Communities" discuss the role of pathogens in natural plant communities. In next two reviews Kamoun deals with the effectors of plant pathogenic oomycetes and Rao discusses our knowledge concerning genome packaging among spherical plant RNA viruses. The review "Quantification and Modelling of Crop Losses" considers the cascade of events that link injuries caused by plant pathogens on crop stands to possible (quantitative and qualitative) crop losses, and to the resulting economic losses. Next review is focus on nonsystemic bunt fungi - *Tilletia indica* and the morphologically similar but distantly related *T. horrida*, considering history, systematics, and biology. Van Loon *et al*. discuss significance of inducible defense-related proteins in infected plants. The review written by Fitt *et al*. considers factors affecting the coexistence of closely related pathogen species on arable crops, with particular reference to data available at Rothamsted for *Septoria tritici/Stagonospora nodorum* (*Mycosphaerella graminicola/Phaeosphaeria nodorum*) (septoria leaf blotch diseases on winter wheat), *Oculimacula yallundae/O. acuformis* (eyespot disease of winter cereals), and *Leptosphaeria maculans/L. biglobosa* (phoma stem canker on winter oilseed rape). The chapter "Virus-Vector Interactions Mediating Nonpersistent and Semipersistent Transmission of Plant Viruses" was written by Ng and Falk. The next review article discusses the improvements made in breeding of *Poa pratensis* for resistance to leaf spot (caused by *Drechslera poae*), stem rust (caused by *Puccinia graminis*), and stripe smut (caused by *Ustilago striiformis*); *Lolium perenne* for resistance to gray leaf spot (caused by *Pyricularia grisea*), stem rust and crown rust (caused by *Puccinia coronata*); *Festuca arundinacea* for resistance to brown patch (*Rhizoctonia solani*) and stem rust; *Agrostis stolonifera* for resistance to dollar spot (caused by *Sclerotinia homoeocarpa*); and *Festuca* spp. for improved disease resistance. The review "Molecular Ecology and Emergence of Tropical Plant Viruses" reveals the role of molecular ecology in unravelling the factors responsible for the emergence of several of the economically most important tropical plant viruses: *Rice yellow mottle virus* (RYMV), *Cassava mosaic geminiviruses* (CMGs), *Maize streak virus* (MSV), and *Banana streak virus* (BSV). Mechanisms involved were recombination and synergism between virus species, new vector biotypes, genome integration of the virus, host adaptation, and long-distance dispersal.

The readers of Biologia Plantarum will be also interested in the reviews "Biology of Flower-Infecting Fungi", "A Model Plant Pathogen from the Kingdom Animalia: Heterodera glycines, the Soybean Cyst Nematode", "Comparative Genomics Reveals What Makes An Enterobacterial Plant Pathogen", "The Dawn of Fungal Pathogen Genomics" and "Fitness of Human Enteric Pathogens on Plants and Implications for Food Safety".

Other reviews covered in this volume are: "The Role of Ethylene in Host-Pathogen Interactions", "Phenazine Compounds in Fluorescent *Pseudomonas* spp. Biosynthesis and Regulation", "Long-Distance RNA-RNA Interactions in Plant Virus Gene Expression and Replication", "Evolution of Plant Pathogenicity in *Streptomyces*" and "Climate Change Effects on Plant Disease: Genomes to Ecosystems".

This book represents a valuable source of up to date information on diverse fields of phytopathology and mechanisms involved in plant-pathogen communication. The reviews are well documented with colour illustrations, figures, diagrams and photographs facilitating the understanding of the presented facts. "Annual Review of Phytopathology" is very useful for scientists and students interested in phytopathology and other fields of plant biology.

M. ŠINDELÁŘOVÁ (*Praha*)